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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Brain tissue is highly heterogeneous with different functions localized in specific areas. We compared the markers of oxidative damage (lipid peroxidation and protein oxidation) and antioxidant status (catalase activity) in frozen brain samples from the cerebellum and frontal, temporal, parietal and occipital cortex of autistic and age-matched normal subjects. In autism, a significant increase in protein oxidation was observed in frontal cortex, temporal cortex, and cerebellum. In the cerebellum and temporal cortex in autism, lipid peroxidation was also significantly increased and catalase activity involved in antioxidant defense was significantly decreased. In contrast, no significant change in above oxidative stress markers was observed in other brain regions between autism and control groups. Since increased oxidative stress in autism can affect the activities of membrane-bound enzymes, namely Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase that play important roles in signal transduction, we compared their activities in autism and controls. Brain-region specific increases in the activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase was observed in the cerebellum and frontal cortex in autism (Ji etal. Life Sci. in press). In contrast, in other regions, i.e., the temporal, parietal and occipital cortices, the activities of these enzymes were similar in autism and control groups. These results suggest that oxidative stress differentially affects selective regions of the brain, i.e. cerebellum, frontal cortex and temporal cortex in autism, and that altered activities of membrane-bound enzymes may contribute to abnormal neuronal circuit functioning in this disease.

15. SUBJECT TERMS
Oxidative stress in autistic brain, lipid peroxidation, protein oxidation

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SUBPROJECT 3

Oxidative damage and inflammation in the brains of autistic subjects: Correlation with severity and phenotypes.

PI: Abha Chauhan, Ph.D.

INTRODUCTION

While the cause of autism remains elusive, autism is considered a multifactorial disorder that is influenced by genetic and environmental factors. Accumulating evidence suggests that oxidative stress may provide a link between susceptibility genes and pre- and post-natal environmental risk agents in the pathophysiology of autism [reviewed in 1]. Under normal conditions**,** a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the anti-oxidant capacity of the cell. These ROS are highly toxic, and if not removed or neutralized, they react with lipids, proteins and nucleic acids and damage membrane properties and cellular functions. Oxidative stress is known to be associated with premature aging of cells and can lead to inflammation, damaged cell membranes, autoimmunity and cell death. The brain is highly vulnerable to oxidative stress due to its limited antioxidant capacity, higher energy requirement and high amounts of unsaturated lipids and iron [2]. The brain makes up about 2% of body mass but consumes 20% of metabolic oxygen. The vast majority of energy is used by the neurons [3]. Glutathione is the most important antioxidant for detoxification and elimination of environmental toxins. Due to the lack of glutathione-producing capacity by neurons, the brain has a limited capacity to detoxify ROS. Therefore, neurons are the first cells to be affected by the increase in ROS and shortage of antioxidants and, as a result, they are most susceptible to oxidative stress. Antioxidants are required for neuronal survival during the early critical period [4]. Children are more vulnerable than adults to oxidative stress because of their naturally low glutathione levels from conception through infancy [5,6]. The risk created by this natural deficit in detoxification capacity in infants is increased by the fact that some environmental factors that induce oxidative stress are found at higher concentrations in developing infants than in their mothers, and accumulate in the placenta.

We have reported that levels of malonyldialdehyde (MDA), a marker of lipid peroxidation (LPO), are increased in the plasma from children with autism [7]. Other studies on erythrocytes and urine samples have also indicated increased levels of lipid peroxidation markers in autism, thus confirming an increased oxidative stress in autism [8,9]. Brain tissue is highly heterogeneous with specific functions localized in specific areas of brain. The studies on oxidative stress in relation to specific regions of brains are lacking in autism. In this study, we analysed the status of lipid peroxidation, protein oxidation and anti-oxidant enzyme in different brain regions (cerebellum, frontal, temporal, occipital and parietal cortex) from autism and control subjects. Since increased oxidative stress in autism can affect the activities of membrane-bound enzymes, such as Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase that are known to maintain intracellular gradients of ions essential for signal transduction, we also compared the activities of these enzymes in different brain regions from autism and control subjects.

BODY

There is lack of knowledge of the causative factors and secondary abnormalities in biochemical pathways in autism. The levels of markers of oxidative damage and antioxidant status were evaluated in homogenates from different brain regions of autistic and control subjects. The postmortem frozen brain samples from the cerebellum and frontal, temporal, parietal and occipital cortex from autistic subjects with age range of 4 to 39 yrs (N = 7–10 for different tissues) and age-matched normal subjects (N = 9–10) were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland. All brain samples were stored at –70˚C. The brain tissues were homogenized in cold buffer containing 25 mM Tris-HCl (pH 7.4), 2 mM EDTA and protease cocktail inhibitor. The protein content of the samples was determined by using the Bio-Rad protein dye assay reagent. We compared the status of lipid peroxidation, protein oxidation and the activities of catalase (enzyme involved in antioxidant defense) and of membrane-bound enzymes (Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase) in the homogenates from different regions of the brain from autism and control subjects. Our results suggest increased oxidative damage in the cerebellum, frontal cortex and temporal cortex but not in occipital and parietal cortex in autism.

(a) Increased lipid peroxidation (LPO): *This study was presented at the International Meeting for Autism Research (May, 2009)[Appendix 1, Ref. 10]*. Lipid peroxidation is a chain reaction between polyunsaturated fatty acids and ROS producing lipid peroxides and hydrocarbon polymers that are both highly toxic to the cell. It is one of the prime mechanisms involved in cellular damage caused by free radicals. LPO is associated with a progressive loss in membrane fluidity, reduction in membrane potential, increase in membrane permeability to ions, which finally leads to cellular damage. MDA is the most abundant toxic aldehyde formed from LPO reaction. The status of lipid peroxidation in the brain homogenates from autism and control subjects was monitored by measuring the contents of MDA, an end product and marker of lipid peroxidation as described previously **[7].** MDA reacts with thiobarbituric acid (TBA) to form a colored complex with a maximal absorbance at 532 nm. MDA levels were significantly increased by 124 % in the cerebellum and by 256 % in the temporal cortex in autism as compared to control subjects (Figs. 1, 2, 3). No overlap of MDA levels was observed in the temporal cortex between autism and control groups (Fig. 2). In the cerebellum, 57 % of autism subjects had MDA levels above the cutoff value (upper range for control group) (Fig. 1). In contrast, no significant change in MDA levels was observed in frontal, occipital and parietal cortex between autism and control groups (Fig. 2, 3).

(b) Increased protein oxidation: *This study was presented at the International Society for Neurochemistry Meeting (2009)[Appendix 2, Ref. 11).* Protein oxidation is defined as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary by-products of oxidative stress. Protein carbonyl derivatives of amino acids (pro, arg, lys, thr) are the most common products of protein oxidation. Reaction of ROS with proteins results in the formation of protein carbonyls. These oxidative modifications cause the loss of structural or catalytic function in the affected protein and contribute to serious deleterious effects on cellular functions. Protein oxidation was assessed in the homogenates from different brain regions of autism and control subjects by quantitation of protein carbonyls using ELISA kit (Cell

Biolab). The method is based on the derivitization of the carbonyl group with dinitrophenylhydrazine (DNPH) to DNP hydrazone, which is probed with anti-DNP antibody. The levels of protein carbonyl in autism were significantly increased by 123 % in frontal cortex, by 108 % in temporal cortex, and by 100 % in cerebellum as compared with controls (Figs. 4,5,6). On the other hand, its levels in parietal and occipital cortex were similar between autism and control groups (Fig. 5,6).

(c) Reduced antioxidant activity. Catalase is one of the primary enzymes involved in the antioxidant defense against oxygen stress. It converts hydrogen peroxide to water and molecular oxygen, thereby reducing the amount of free hydroxyl radical formation ($2H_2O_2 \rightarrow O_2 + 2H_2O$) [12]. Catalase activity was compared in the brain homogenates from autistic and control subjects. Its activity was significantly decreased in the cerebellum ($p=0.0007$) and temporal cortex ($p=0.0122$) in autistic group compared with control group. However, its activity was similar in frontal cortex between autism and control groups. (Fig. 7).

(d) Increased activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase *[Appendix 3, Ref. 13]*. Since we observed that lipid peroxidation is increased in autistic brain, we also studied if oxidative stress can affect the activities of membrane-bound enzymes, namely Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in different brain regions of autistic subjects. The activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were assessed in the brain homogenates by measuring inorganic phosphorus released by the action of Na^+/K^+- and Ca^{2+}/Mg^{2+}- dependent hydrolysis of ATP. Our results showed that the activities of both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were significantly increased in the cerebellum in autism as compared with age-matched controls, while the activity of Na^+/K^+-ATPase was also significantly increased in the frontal cortex in autism. In other regions of cerebrum i.e., occipital, parietal and temporal cortex, the activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were similar between autistic and control subjects.

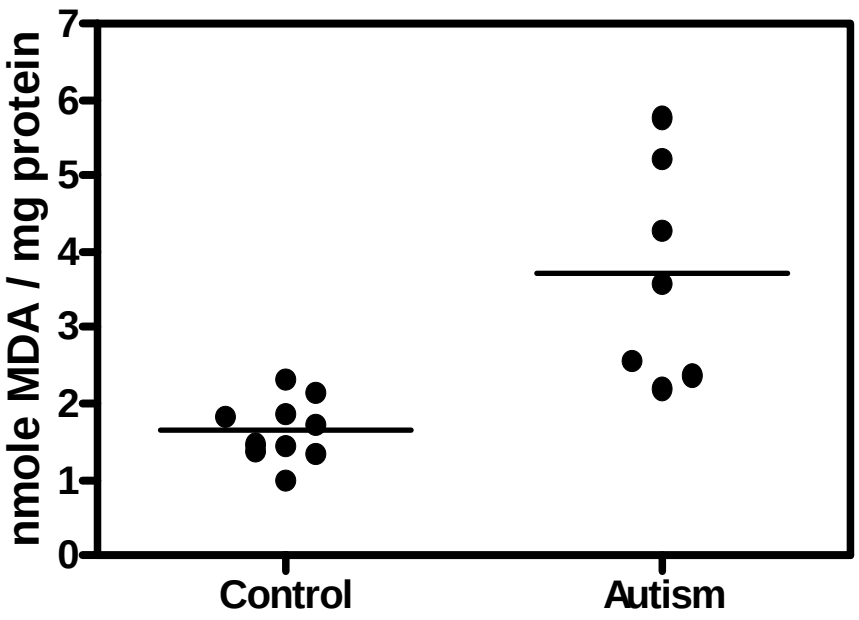

Fig. 1. Scattered plot showing increased lipid peroxidation in the cerebellum from autism subjects in comparison with control subjects. Lipid peroxidation was measured by quantitating MDA contents (nmoles/mg protein) in brain homogenates.

• *MDA levels in cerebellum were significantly higher in autism (Mean ± SE = 3.71± 0.54 , Range= 2.19-5.77, N=7, p= 0.0005, unpaired t-test; p= 0.0042, paired t-test) as compared to control subjects (Mean ± SE = 1.65 ± 0.13, Range= 1.00-2.32, N=10).*

• *There was a 124 % increase in cerebellar levels of MDA in autism vs. controls.*

• *Four of seven (i.e. 57 %) autism subjects had MDA levels in cerebellum above the upper cutoff value of range for control group.*

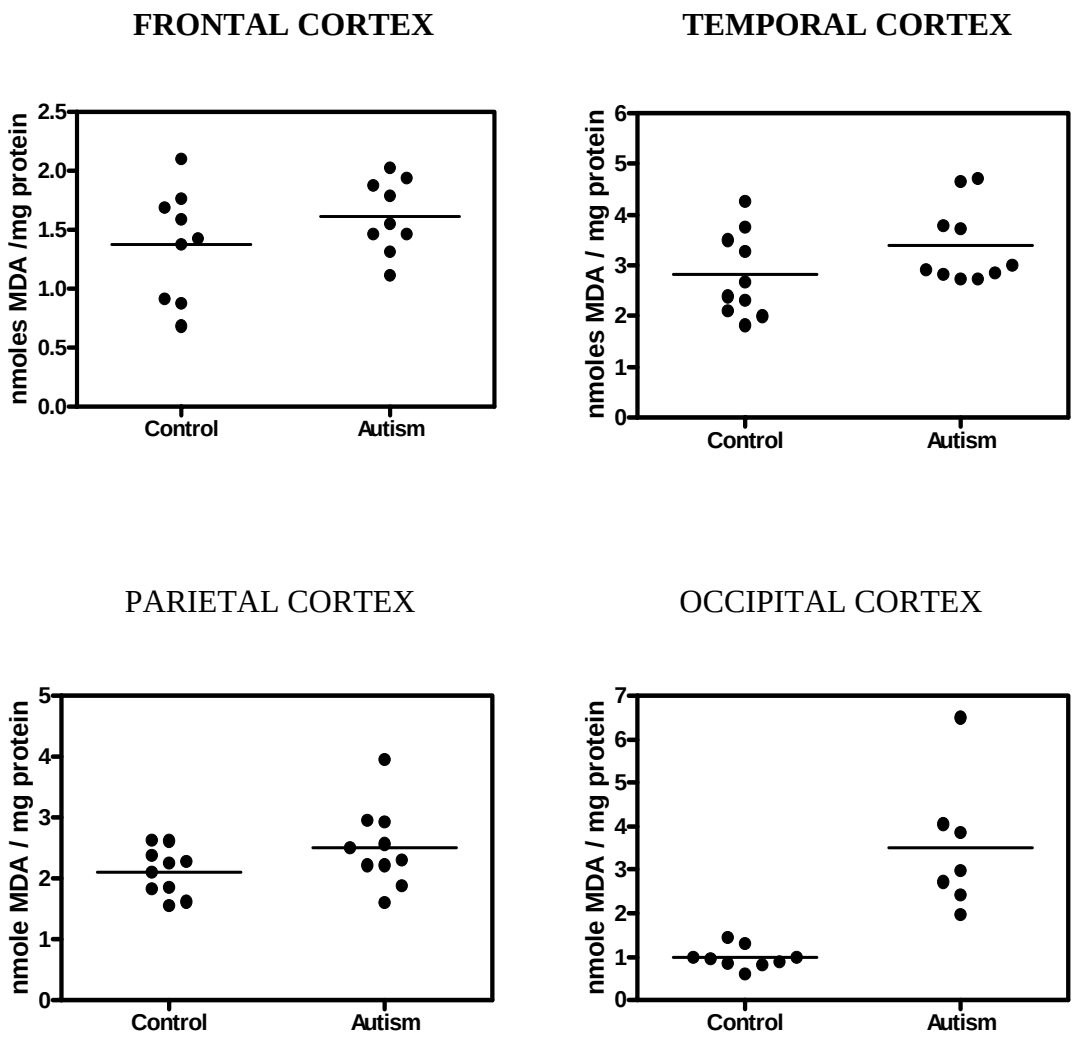

Fig. 2. Lipid peroxidation in different regions of cerebrum i.e. frontal, temporal, parietal and occipital cortex in autism and control groups.

- *A 256 % increase in MDA levels (p=0.0002, unpaired t-test, paired t-test) was observed in the temporal cortex in autism (Mean ± SE =3.49 ± 0.57, N=7) as compared to control subjects (Mean ± SE =0.98 ± 0.09, N=9).*

- *There was no overlap of MDA levels in the temporal cortex between autism (Range: 1.96-6.50) and control groups (Range: 0.59-1.44).*

- *No significant change in MDA levels was observed in frontal, occipital and parietal cortex between autism and control groups.*

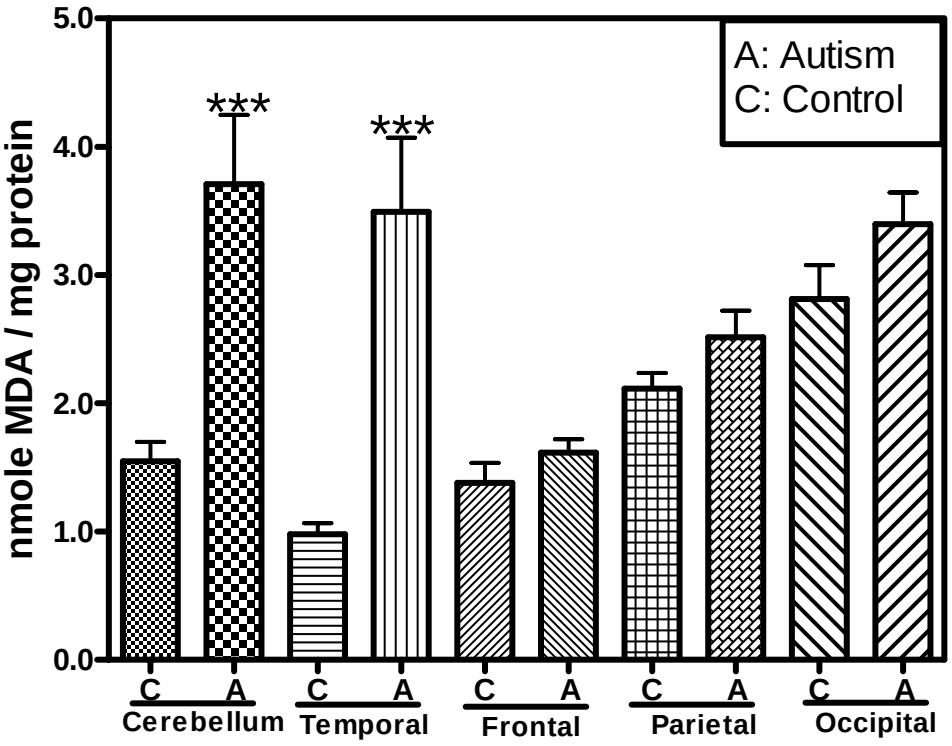

Fig. 3. Comparison of lipid peroxidation in different brain regions of autism and control subjects.

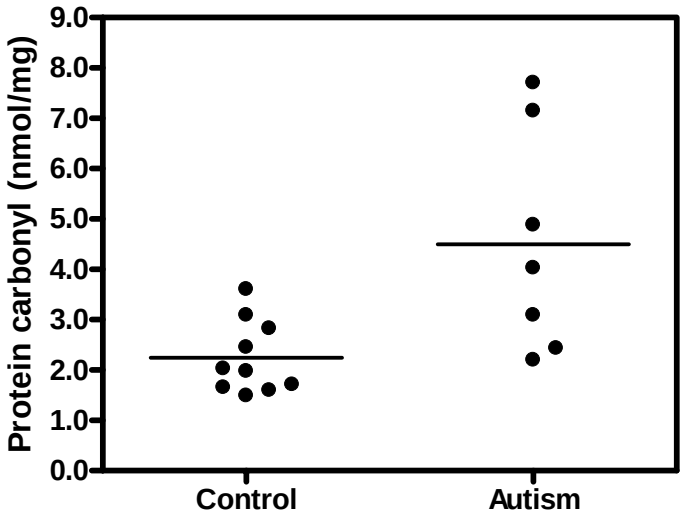

Fig. 4. Scattered plot showing increased protein oxidation in the cerebellum from autism subjects in comparison with control subjects. Protein oxidation was measured by quantitating protein carbonyls (nmoles/mg) in brain homogenates.

- *Protein carbonyl levels in cerebellum were significantly higher in autism (Mean ± SE = 4.50± 0.83 , Range= 2.20-7.70, N=7, p= 0.0082, unpaired t-test; p= 0.0425, paired t-test) as compared to control subjects (Mean ± SE = 2.24 ± 0.23, Range= 1.49-3.60, N=10).*

- *There was a 100 % increase in cerebellar levels of protein carbonyl in autism vs. controls.*

- *Four of seven (i.e. 57 %) autism subjects had protein carbonyl levels in cerebellum above the upper cutoff value of range for control group.*

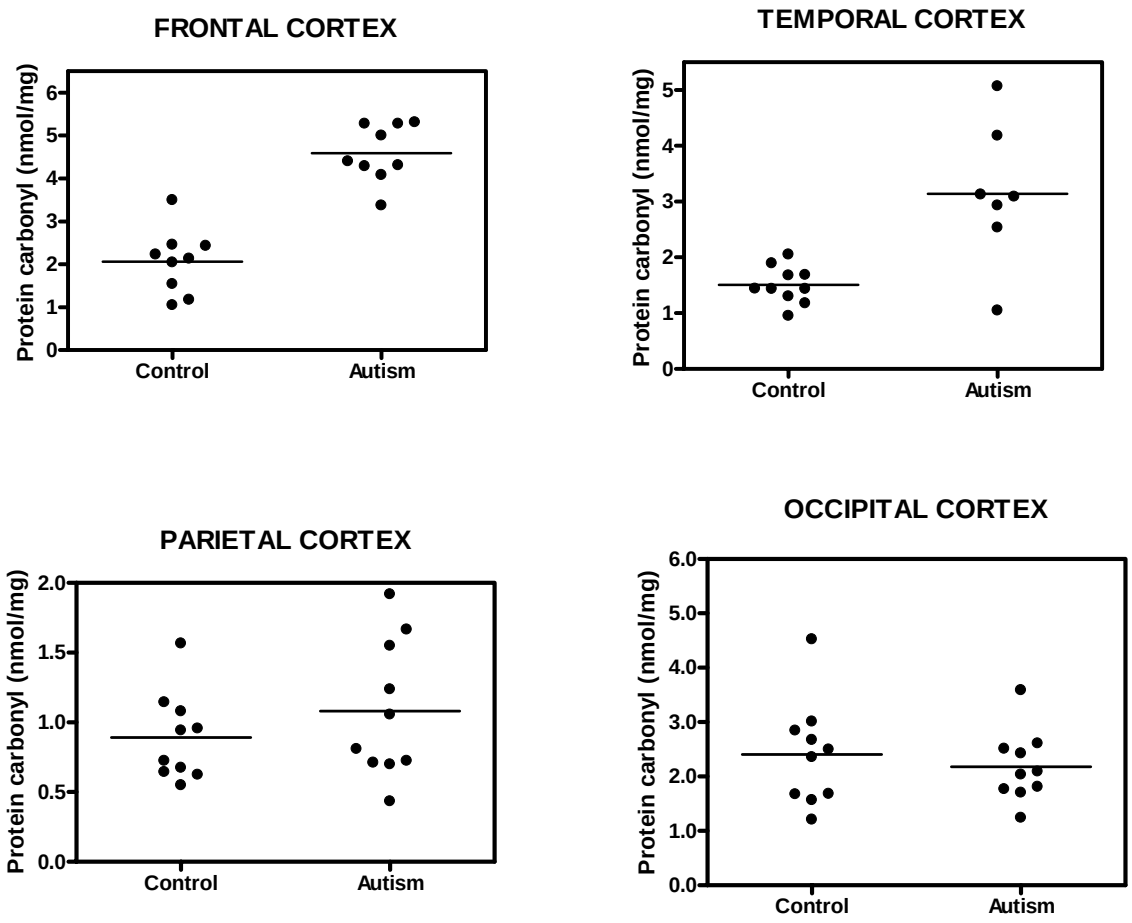

Fig. 5. Protein oxidation in different regions of cerebrum i.e. frontal, temporal, parietal and occipital cortex in autism and control groups.

- *A 123 % increase in protein carbonyl levels (p=0.0001, unpaired t-test, paired t-test) was observed in the frontal cortex in autism (Mean ± SE =4.60 ± 0.22, N=9) as compared to control subjects (Mean ± SE =2.06 ± 0.25, N=9).*

- *A 108 % increase in protein carbonyl levels (p=0.0013, unpaired t-test; p=0.0137, paired t-test) was observed in the temporal cortex in autism (Mean ± SE =3.14 ± 0.48, N=7) as compared to control subjects (Mean ± SE =1.51 ± 0.10, N=10).*

- *There was no or minimal overlap of protein carbonyl levels in the frontal and temporal cortex between autism and control groups.*

- *No significant change in MDA levels was observed in frontal, occipital and parietal cortex between autism and control groups.*

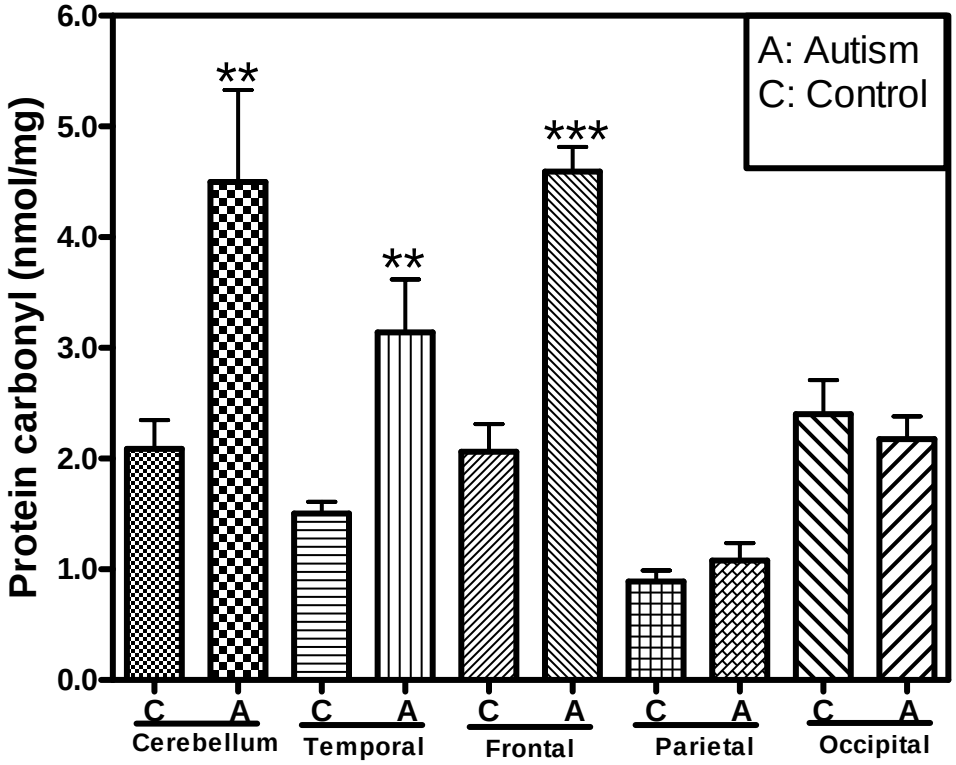

Fig. 6. Comparison of protein oxidation in different brain regions of autism and control subjects.

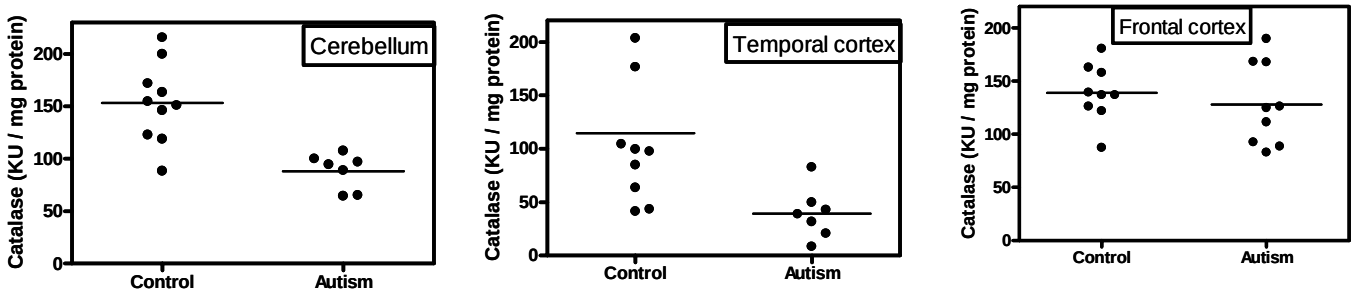

Fig. 7. Comparison of catalase activity in different brain regions of autism and control subjects.

• *Catalase activity was significantly decreased (p=0.0007, unpaired t-test) in the cerebellum in autism (Mean ± SE =88.15 ± 6.40, N=7) as compared to control subjects (Mean ± SE =153.2 ± 11.98, N=10).*

• *A significant decrease in catalase activity (p=0.0122, unpaired t-test) was also observed in the temporal cortex in autism (Mean ± SE =39.14 ± 8.96, N=7) as compared to control subjects (Mean ± SE =114.5 ± 21.01, N=9).*

• *No significant change in catalase activity was observed in the frontal cortex between autism and control groups.*

KEY RESEARCH ACCOMPLISHMENTS

- Lipid peroxidation was significantly increased by 124 % in the cerebellum, and by 256 % in the temporal cortex in autism as compared to control subjects. No overlap of MDA levels was observed in the temporal cortex between autism and control groups. In the cerebellum, 57 % of autism subjects had MDA levels above the cutoff value (upper range for control group). In contrast, no significant change in lipid peroxidation was observed in frontal, occipital and parietal cortex between autism and control groups.

- The levels of protein carbonyl in autism were increased by 123 % in frontal cortex, by 108 % in temporal cortex, and by 100 % in cerebellum from autistic subjects as compared with controls. There was no or minimum overlap of protein carbonyl levels in the frontal and temporal cortex between autism and control groups. In the cerebellum, 57 % of autism subjects had protein carbonyl levels above the cutoff value (upper range for control group). On the other hand, its levels in parietal and occipital cortex were similar between autism and control groups.

- The activity of catalase involved in antioxidant defense was significantly lower in cerebellum and temporal cortex in autistic subjects as compared with control subjects.

- The activities of both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase (membrane-bound enzymes) were significantly increased in the cerebellum in the autistic samples compared with their age-matched controls. The activity of Na^+/K^+-ATPase but not Ca^{2+}/Mg^{2+}-ATPase was also significantly increased in the frontal cortex of the autistic samples as compared to the age-matched controls. In contrast, in other regions, i.e., the temporal, parietal and occipital cortices, the activities of these enzymes were similar in autism and control groups.

Our results indicate the presence of oxidative stress in the specific regions of brain i.e., cerebellum, frontal and temporal cortex of autistic individuals compared with brain samples from age-matched control subjects. The results of this study also indicate brain-region specific increases in the activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in autism.

REPORTABLE OUTCOMES

Abstracts

1. A. Chauhan, B. Muthaiyah, M.M. Essa,W. T. Brown, J.Wegiel and V. Chauhan. Increased lipid peroxidation in cerebellum and temporal cortex in autism. Presented at the International Meeting of Autism Research (IMFAR), May 2009.

2. V. Chauhan, B. Muthaiyah, M.M. Essa**,** J.Wegiel, W. T. Brown, A. Chauhan. Increased lipid and protein oxidation in the brain of autistic individuals. J. Neurochem. 110 (Suppl. 2), 33 (Sept 2009).

Publications

1. Ji, L., Chauhan, A., Brown, W.T. and Chauhan, V. Increased activities of Na/K-ATPase and Ca/Mg-ATPase in the frontal cortex and cerebellum of autistic individuals. Life Sci. (in press).

CONCLUSIONS

Brain is a heterogeneous organ where specific functions are attributed to specific regions. Our results suggest that autism is associated with increased oxidative stress in the brain. We observed increased lipid peroxidation and increased protein oxidation coupled with reduced antioxidant activity in postmortem brain samples from autistic subjects compared with control subjects. Our results also indicate that oxidative stress differentially affects selective regions of the brain, i.e. cerebellum, frontal cortex and temporal cortex, rather than generalised oxidative damage in autism. Increased oxidative damage may also lead to inflammation because oxidative stress serves as a major upstream component in the signaling cascade involved in activation of redox-sensitive transcription factors and pro-inflammatory gene expression resulting in an inflammatory response.

K^+, Na^+ and Ca^{2+} play important roles in neuronal signaling due to conduction of electrical activity of neurons [14]. Therefore, control of excitability of neurons is maintained by the ionic environment. Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are two ATP-hydrolyzing enzymes which maintain the electrochemical gradient in the cells in an energy-dependent manner. The activities of both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were significantly increased in the cerebellum in autism as compared with age-matched controls, while the activity of Na^+/K^+-ATPase was also significantly increased in the frontal cortex in autism. The activities of these enzymes were similar between autistic and control subjects in other brain regions i.e., occipital, parietal and temporal cortex. Since K^+, Na^+ and Ca^{2+} play important roles in developing electrochemical gradients and in neuronal signaling, the altered activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase may have a significant impact on brain function in individuals with autism [Appendix 3, Ref. 13].

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APPENDICES

1. A. Chauhan, B. Muthaiyah, M.M. Essa,W. T. Brown, J. Wegiel and V. Chauhan. Increased lipid peroxidation in cerebellum and temporal cortex in autism. Presented at the International Meeting of Autism Research (IMFAR), May 2009 (Abstract).

2. V. Chauhan, B. Muthaiyah, M.M. Essa**,** J. Wegiel, W. T. Brown and A. Chauhan. Increased lipid and protein oxidation in the brain of autistic individuals. Presented at the International Society for Neurochemistry Meeting, J. Neurochem. 110 (Suppl. 2), 33 (Sept 2009).

3. L. Ji, A. Chauhan,W. T. Brown and V. Chauhan. Increased activities of Na/K-ATPase and Ca/Mg-ATPase in the frontal cortex and cerebellum of autistic individuals. Life Sci. (in press).

APPENDIX 1

Presented at the International Meeting of Autism Research (IMFAR), May 2009

Increased lipid peroxidation in cerebellum and temporal cortex of brain in autism

Abha Chauhan, Balu Muthaiyah, Mohamed MM Essa,W. Ted Brown, Jerzy Wegiel and Ved Chauhan

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Background: Accumulating evidence from our and other groups suggests that oxidative stress may provide a link between susceptibility genes and pre- and post-natal environment stressors in the pathophysiology of autism. Brain tissue is highly heterogeneous with different functions localized in specific areas. Studies on oxidative stress in relation to specific regions of brain are lacking in autism.
Objectives: In this study, the status of lipid peroxidation was compared in postmortem brain samples from the cerebellum and frontal, temporal, parietal and occipital cortex from 4-39 year-old autistic subjects (n of 7-10 for different tissues) and age-matched normal subjects (n of 9-10).
Methods: The levels of malonyldialdehyde (MDA), an end product of fatty acid oxidation, were assayed in the brain homogenates from autism and control subjects.
Results: MDA levels were significantly increased in the cerebellum by 124 % and temporal cortex by 256 % in autism as compared to control subjects. No overlap of MDA levels was observed in the temporal cortex between autism and control groups. In cerebellum, 57 % autism subjects had MDA levels above the cutoff value (upper range for control group). In contrast, no significant change in MDA levels was observed in frontal, occipital and parietal cortex between autism and control groups.
Conclusion: These results suggest that oxidative stress differentially affects selective regions of the brain, i.e. cerebellum and temporal cortex, in autism.
Acknowledgements: This work was supported by NYS OMRDD, and by research grants from Department of Defense, Autism Speaks, Autism Research Institute.

APPENDIX 2

Abstract published: J. Neurochem. 110 (Suppl. 2), 33 (Sept 2009).
Presented at the International Society for Neurochemistry Meeting (2009).

Increased lipid and protein oxidation in the brain of autistic individuals

V. Chauhan, B. Muthaiyah, M.M. Essa**,** J. Wegiel, W. T. Brown, A. Chauhan

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Accumulating evidence suggests that oxidative stress may provide a link between susceptibility genes and pre- and post-natal environmental stressors in the pathophysiology of autism. Brain tissue is highly heterogeneous with different functions localized in specific areas. Studies on oxidative stress in relation to specific regions of the brain are lacking in autism. We compared the status of lipid peroxidation and protein oxidation in postmortem brain samples from the cerebellum and frontal, temporal, parietal and occipital cortex from autistic subjects with age range of 4 to 39 yrs (N = 7–10 for different tissues) and age-matched normal subjects (N = 9–10). Protein oxidation, assessed by quantitation of protein carbonyls, was significantly increased in autism by 123 % in frontal cortex, by 108 % in temporal cortex, and by 100 % in cerebellum as compared with controls. On the other hand, its levels in parietal and occipital cortex were similar between autism and control groups. In addition, the levels of malonyldialdehyde (MDA), a marker of lipid peroxidation, were significantly increased by 124 % in the cerebellum and by 256 % in the temporal cortex in autism as compared with control subjects. In contrast, no significant change in MDA levels was observed in frontal, occipital and parietal cortex between autism and control groups. These results suggest that oxidative stress differentially affects selective regions of the brain, i.e. cerebellum, frontal cortex and temporal cortex in autism.

APPENDIX 3

Life Sci. (in press)

Increased activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in the frontal cortex and cerebellum of autistic individuals

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ABSTRACT

Aims

Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are enzymes known to maintain intracellular gradients of ions that are essential for signal transduction. The aim of this study was to compare the activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in post-mortem brain samples from the cerebellum and frontal, temporal, parietal, and occipital cortices from autistic and age-matched control subjects.

Main methods

The frozen postmortem tissues from different brain regions of autistic and control subjects were homogenized. The activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were assessed in the brain homogenates by measuring inorganic phosphorus released by the action of Na^+/K^+- and Ca^{2+}/Mg^{2+}- dependent hydrolysis of ATP.

Key findings

In the cerebellum, the activities of both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were significantly increased in the autistic samples compared with their age-matched controls. The activity of Na^+/K^+-ATPase but not Ca^{2+}/Mg^{2+}-ATPase was also significantly increased in the frontal cortex of the autistic samples as compared to the age-matched controls. In contrast, in other regions, i.e., the temporal, parietal and occipital cortices, the activities of these enzymes were similar in autism and control groups.

Significance

The results of this study suggest brain-region specific increases in the activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in autism. Increased activity of these enzymes in the frontal cortex and cerebellum may be due to compensatory responses to increased

intracellular calcium concentration in autism. We suggest that altered activities of these enzymes may contribute to abnormal neuronal circuit functioning in autism.

INTRODUCTION

Autism is a severe neurological disorder that causes impairment in language, cognition and socialization (Lord et al. 2000). It is a heterogeneous disorder, both etiologically and phenotypically. Autism belongs to a group of neurodevelopmental disorders known as autism spectrum disorders (ASD) that includes Pervasive Developmental Disorder- Not Otherwise Specified (PDD-NOS), Asperger disorder, Childhood Disintegrative Disorder (CDD) and Rett syndrome. According to the Centers for Disease Control (CDC), 1 in 150 children is diagnosed with ASD.

Genetic, neurochemical, neuroimaging and behavioral studies suggest that neural properties may be perturbed in autism, giving rise to abnormalities in processing of neuronal information leading to complex behavioral abnormalities (Belmonte et al., 2004). Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are known to play important roles in neuronal transmission. A gradient of high K^+ and low Na^+ intracellular concentration is needed for the optimum neuronal functions (McCormick and Huguenard, 1994, Palladina et al., 2005). Na^+/K^+-ATPase is a membrane-bound enzyme involved in maintaining the Na^+ and K^+ gradient across the cell membrane. It is ubiquitously expressed in neurons (Pietrini et al., 1992), and helps maintain normal neuronal function. The Na^+/K^+-ATPase extrudes three Na^+ ions and imports two K^+ ions. This activity is important in the regulation of membrane potential. The Na^+/K^+-ATPase activity contributes to the resting

membrane potential in the cell, and returns Na^+ and K^+ concentrations to their resting transmembrane levels after bursts of stimulatory activity (Blaustein, 1993). Na^+/K^+-ATPase abnormality has been reported to be involved in several neurological diseases such as seizures (Brines et al., 1995; Fernandes et al., 1996b), bipolar disorder (Amiet et al., 2008; Christo and el Mallakh, 1993), spongiform encephalopathy (Renkawek et al., 1992), and Alzheimer's disease (Rose and Valdes, 1994). Na^+/K^+-ATPase may also have implications in behavioral defects. Lingrel et al. (2007) reported that haploinsuffciency of Na^+/K^+-ATPase $\alpha 2$ and $\alpha 3$ isoforms results in behavioral defects.

Calcium is an important signaling molecule in cells (Berridge, 1992). Many cellular functions are regulated by intracellular free calcium concentrations. In resting cells, a sub-optimum concentration of intracellular calcium is maintained either by storing calcium in intracellular reserves by the action of ATPase (Nori et al., 1996), by extrusion of calcium by plasma membrane-bound calcium ATPase (Carafoli et al., 1996) or by Na^+/ Ca^{2+} exchange (Blaustein and Lederer, 1999). In stimulated cells, a sudden influx of calcium occurs in a receptor-coupled manner where calcium participates in activating several proteins, which perform specific functions. Neurons use intracellular Ca^{2+} to control various functions. Disturbances in Ca^{2+} homeostasis can lead to neuronal dysfunction and eventual neuronal death. Several neurological diseases are caused primarily by malfunctioning of Ca^{2+} channels or Ca^{2+}/Mg^{2+}-ATPase (Cooper and Jan, 1999; Jacobsen et al., 1999). Recently, Gargus reported genetic calcium signaling abnormalities in several neurological conditions, including seizures, migranes and autism (Gargus, 2009). However, it is not known whether Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase abnormalities are involved in autism. The present study was undertaken to

determine whether the activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are affected in autism. We found that activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are specifically increased in the frontal cortex and cerebellum of brains from autistic subjects, while they were unchanged in the parietal, occipital and temporal cortices.

MATERIALS AND METHODS

Materials. Samples of postmortem frozen brain regions, i.e., the cerebellum, and cortices from the frontal, temporal, parietal and occipital lobes (N= 6-10 for different brain regions) from autistic and age-matched control subjects (N= 8-10) were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders. The age (Mean ± S.E.) for autistic subjects was 13 ± 3.7 y and for control subjects, 12.5 ± 3.5 y. The mean postmortem interval (PMI) for the autistic samples was 22 ± 4.5 h, and for control samples, 17± 1.3 h. Donors with autism fit the diagnostic criteria of the Diagnostic and Statistical Manual-IV, as confirmed by the Autism Diagnostic Interview-Revised (ADI-R). All brain samples were stored at -70 ^{0}C. This study was approved by the Institutional Review Board of the New York State Institute for Basic Research.

Preparation of homogenates. The tissues were homogenized (10% w/v) in cold buffer containing 50 mM Tris-HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM β-mercaptoethanol and protease inhibitor cocktail (Sigma-Aldrich) in a Downs homogenizer with 5 strokes at 4 ^{0}C. The protein concentration was assayed by the BioRad protein assay kit.

Measurement of Na^+/K^+-ATPase activity. The reaction mixture containing 25 µl of 2 M NaCl, 25 µl of 25 mM KCl, 25 µl of 60 mM $MgCl_2$, 5 µl of 10 mM EGTA and brain homogenate (0.5 mg) was adjusted to a total volume of 490 µl with 50 mM Tris-HCl, pH 7.5, and incubated at 37 ^{0}C for 10 min. The reaction was started by adding 10 µl of 150 mM ATP. After 1 h, the reaction was stopped by adding 1 ml of cold 15% TCA. The samples were kept on ice for 1 h, followed by centrifugation at 1,000 g for 15 min. Inorganic phosphorus in the 500 µl supernatant was measured by the method of Fiske and Subbaraw (1953).

Measurement of Ca^{2+}/Mg^{2+}-ATPase activity. The reaction mixture containing 25 µl of 2 M NaCl, 25 µl of 25 mM KCl, 25 µl of 60 mM $MgCl_2$, 5 µl of 10 mM Quabain, 10 µl of 10 mM $CaCl_2$, and brain homogenate (0.5 mg) was adjusted to a total volume of 490 µl with 50 mM Tris-HCl, pH 7.5, and incubated at 37 ^{0}C for 10 min. The reaction was initiated by adding 10 µl of 150 mM ATP. After 1 h, the reaction was stopped by adding 1 ml of cold 15% TCA. The samples were kept on ice for 1 h, followed by centrifugation at 1,000 g. Inorganic phosphorus in the 500 µl supernatant was then measured.

Statistical analysis. The enzyme activities in autism and control groups were analyzed by unpaired student's t-test.

RESULTS

Na^+/K^+-ATPase activity in different brain regions from autistic and control subjects. Fig. 1 shows the Na^+/K^+-ATPase activity in the cerebellum of autistic and age-matched control subjects. The Na^+/K^+-ATPase activity (μg phosphorus released/mg protein/hr) in the cerebellum of the autistic samples (Mean ± S.E.:188 ± 13) was significantly higher ($p < 0.03$) as compared to control samples (156 ± 8). In Fig. 2, the Na^+/K^+-ATPase activity in the cortices from frontal, temporal, parietal and occipital regions is shown. The activity of Na^+/K^+-ATPase in the frontal cortex of the autistic subjects (169 ± 5) was significantly higher ($p < 0.03$) as compared to the control subjects (145 ± 8.7). However, the activity of the Na^+/K^+-ATPase was similar between the autistic and control groups in other brain regions, i.e., temporal cortex (autism, 142± 13; and controls, 128 ± 7.5); parietal cortex (autism, 131 ± 4; and controls, 130 ± 6); and occipital cortex (autism, 294 ± 22, and controls, 309 ± 15). No relation was observed between PMI and the activity of this enzyme in the brain.

Ca^{2+}/Mg^{2+}-ATPase activity in different brain regions from autistic and control subjects. Ca^{2+}/Mg^{2+}-ATPase activity in the cerebellum of autistic and control subjects is shown in Fig. 3. The Ca^{2+}/Mg^{2+}-ATPase activity (μg phosphorus released/mg protein/hr) in the cerebellum of the autistic samples (Mean ± S.E.: 190 ± 13) was significantly higher ($p < 0.0005$) than in age-matched controls (130 ± 4.5). There was no overlap in the Ca^{2+}/Mg^{2+}-ATPase activity between autistic and control samples.

Fig. 4 shows the Ca^{2+}/Mg^{2+}-ATPase activity in the cortices from frontal, temporal, parietal and occipital regions. No significant differences in the activity of Ca^{2+}/Mg^{2+}-ATPase was observed in frontal cortex (autism, 105 ± 6.7; and controls, 96.7 ± 5.6); temporal cortex (autism, 110± 12; and controls, 98 ± 6); parietal cortex (autism, 111 ± 6, and control, 115 ± 10); and occipital cortex (autism, 211 ± 22; and controls, 200 ± 8). No relation was observed between PMI and this activity of the enzyme in the brain.

DISCUSSION

K^+, Na^+ and Ca^{2+} play important roles in neuronal signaling due to conduction of electrical activity of neurons (McCormick and Huguenard, 1994). Therefore, control of excitability of neurons is maintained by the ionic environment. Intracellular concentrations of Na^+ and Ca^{2+} are several folds lower and that of potassium are higher as compared to their extracellular concentrations. The net transmembrane potential across the membrane is maintained at -60 mV. If the ionic concentration is perturbed (e.g., levels of intracellular Ca^{2+} and Na^+ or extracellular K^+ are altered), this can lead to depolarization and abnormal neuronal activity due to depolarization of neuronal-terminals, neurotransmitters release, depolarization of neurons and discharge of action potential (Somojen, 2002).

Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are two ATP-hydrolyzing enzymes which maintain the electrochemical gradient in the cells in an energy-dependent manner. Na^+/K^+-ATPase extrudes three Na^+ molecules in exchange for internalization of two K^+ molecules. The Na^+/K^+-ATPase is composed of multiple isoforms ($\alpha1$, $\alpha2$ and $\alpha3$), and these isoforms differ in their distribution in tissues and during development. Lingrel et

al. (2007) reported that haploinsuffciency of its α2 and α3 isoforms results in behavioral defects. In another study, mutations in a C-terminal region of other voltage-gated Na^+ channels have been reported to reduce the amount of channel inactivation (Glaaser et al., 2006; Kim et al., 2004). Another report suggests functional deficit of Ca^{2+}-activated K^+ channel (BKCa), a synaptic regulator of neuronal excitability with autism (Laumonnier et al., 2006). Disruption of the BKCa gene (KCNMA1) led to haploinsufficiency and reduced BKCa activity in autism. These reports on decrease in BKCa channel activity, and reduced inactivation of voltage-gated Ca^{2+} channels in individuals with autism, raise the possibility that excessive ion channel activity may lead to ASD.

Since K^+, Na^+ and Ca^{2+} play important roles in developing electrochemical gradients and in neuronal signaling, the altered activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase may have a significant impact on brain function in autistic subjects. Our results show that the activities of both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were significantly increased in the cerebellum in autism as compared with age-matched controls, while the activity of Na^+/K^+-ATPase was also significantly increased in the frontal cortex in autism. In other regions of cerebrum i.e., occipital, parietal and temporal cortex, the activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase were similar between autistic and control subjects. Increased activity of Na^+/K^+-ATPase has been reported in several other pathological conditions such as in experimentally induced epilepsy (Fernandes et al., 1996; Reime et al., 2007), and in Crush syndrome (Desai and Desai, 2007). In chronic fatigue syndrome, the activities of both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase are increased in sarcoplasmic reticulum membranes (Fulle et al., 2003). In addition, Takser et al. (2003) reported a correlation of ATPase activities with

early psychomotor development in humans. Rapid eye movement sleep deprivation has also been reported to increase Na^+/K^+-ATPase activity (Mallick et al., 2000). In addition, certain environmental factors such as lead have been reported to increase the activity of Na^+/K^+-ATPase (Regunathan and Sundaresan, 1985).

After a stimulus, calcium flows rapidly into neurons through various types of membrane channels including voltage-dependent and receptor-coupled channels. Intracellular Ca^{2+} concentrations are quickly restored to resting levels primarily through Ca^{2+}/Mg^{2+}-ATPase, Na^+/Ca^+ exchange, and endoplasmic sequestration. Calcium is essential for neurotransmitter release, and Ca^{2+} influx is essential for neuronal excitability. Improper intracellular regulation of calcium has been linked with several neurological disorders. The receptor-coupled increase in intracellular levels of calcium is important for neuronal survival, differentiation, migration, and synaptogenesis (Aamodt and Constantine-Paton, 1999; Cline, 2001; Komuro and Rakic, 1998; Moody and Bosma, 2005; Represa and Ben Ari, 2005; Spitzer et al., 2004). Defects in these developmental processes can lead to neuroanatomical abnormalities, such as increased cell-packing density, decreased neuron size and arborizations, and alterations in connectivity. Such abnormalities have been associated with ASD patients (Courchesne et al., 2005; DiCicco-Bloom et al., 2006). Plasma membrane calcium-ATPase plays an important role in the translocation of calcium from the cytosol to the extracellular milieu. Our results suggest that Ca^{2+}/Mg^{2+}-ATPase activity is significantly increased in the cerebellum of autistic subjects, but not in other regions of the brain. Although Ca^{2+}/Mg^{2+}-ATPase activity in the frontal cortex of autism subjects was not significantly changed but a trend towards increased Ca^{2+}/Mg^{2+}-ATPase activity was observed as compared to controls. The median

Ca^{2+}/Mg^{2+}-ATPase activity in the frontal cortex for autism was 113 μg phosphorus released/mg protein/hr while for control group, it was 99 μg phosphorus released/mg protein/hr. A differential effect of ATPase activity in different regions of brain is not unique. In epilepsy, the intrasynaptosomal Ca^{2+}/Mg^{2+}-ATPase activity was reported to be decreased in the hippocampus, but not in the temporal cortex (Nagy et al., 1990).

Voltage-gated calcium channels mediate calcium influx in response to membrane depolarization and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression. Their activity is essential for coupling electrical signals on the cell surface to physiological events in cells. Functional mutations in genes encoding voltage-gated Ca^{2+} channels have been suggested as a possible cause of ASD (Hemara-Wahanui et al., 2005b; Splawski et al., 2006; Splawski et al., 2004). Point mutations in the gene encoding the L-type voltage-gated Ca^{2+}channel CaV1.2 (CACNA1C) cause Timothy syndrome, a multisystem disorder that includes cardiac abnormalities and autism (Splawski et al., 2005; Splawski et al., 2004). CaV1.2 plays an important role in the activation of transcription factors, such as cAMP response-element-binding protein (CREB) and myocyte enhancer factor 2 (MEF2), involving neuronal survival and dendritic arborization (West et al., 2001). The mutations associated with Timothy syndrome prevent voltage-dependent inactivation of CaV1.2, which causes the channels to remain open longer and allow the influx of more Ca^{2+} than wild-type channels (Splawski et al., 2005; Splawski et al., 2004) leading to increased intracellular Ca^{2+}. Additional evidence of calcium's involvement in autism comes from a mutation identified in the CACNA1F gene, which encodes the L-type voltage-gated Ca^{2+} channel, CaV1.4. This mutation was reported to cause autistic symptoms in a New Zealand family

where the affected subjects have stationary night blindness (Hemara-Wahanui et al., 2005a; Hope et al., 2005). ASD-associated mutations have been identified not only in genes encoding Ca^{2+} channels themselves but also in genes encoding ion channels whose activity is directly modulated by Ca^{2+} such as Ca^{2+}-dependent Na^{+} channels. Several point mutations in SCN1A and SCN2A genes, which encode the voltage-activated Na^{+} channels NaV1.1 and NaV1.2 respectively has been reported (Kamiya et al., 2004; Weiss et al., 2003).

Wingless-type mouse mammary tumor virus (MMTV) integration site member (Wnt) proteins are known to form a family of highly conserved and secreted signaling molecules, which regulate cell-to-cell interactions during embryogenesis. The role of WNT2 has been implicated in ASD. Two families with mutations in WNT2 have been identified, and a polymorphism in an upstream region of WNT2 has been associated with families characterized with severe language abnormalities (Wassink et al., 2001). Increase in Ca^{2+} concentration has been reported to enhance the synthesis and release of Wnt through the activity of the Ca^{2+}-regulated transcription factor CREB (Wayman et al., 2006). Because of the pivotal role of calcium in cellular signaling, calcium may play an important role in the etiology of ASD.

The increased activity of Ca^{2+}/Mg^{2+}-ATPase in the cerebellum of autistic subjects may be attributable to several factors. Ca^{2+}/Mg^{2+}-ATPase activity may increase due to compensatory mechanisms in response to increased intracellular calcium levels in autism. Heguilen et al. (2009) reported increases in Ca^{2+}/Mg^{2+}-ATPase activity in patients with hypercalciuric nephrolithiasis. In addition, Ca^{2+}/Mg^{2+}-ATPase activity can also be activated by lysophosphatidylcholine, a phospholipase A_2 (PLA_2)–mediated lipolytic

product in the membrane. It has also been reported that the levels of polyunsaturated fatty acids, another lipolytic product of PLA_2, are decreased in the erythrocyte membranes of autistic subjects as compared with normal control subjects (Bell et al., 2000). Increased activity of PLA_2, an enzyme that removes unsaturated fatty acids from phospholipids, has also been reported in erythrocytes from autistic subjects (Bell et al., 2004). Additionally, increased levels of phospholipase A_2 have been observed in the erythrocytes of patients with schizophrenia (Ward, 2000) and dyslexia (MacDonell et al., 2000). Since chromosomal linkage studies in autism point to a locus which includes the PLA_2 gene (Lamb et al., 2000), this enzyme may also have an important role in the etiology of autism. In conclusion, Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities in autism may be increased in response to increased intracellular calcium concentration, and may contribute to altered neocotical circuitry in the cerebellum and frontal cortex of individual with autism.

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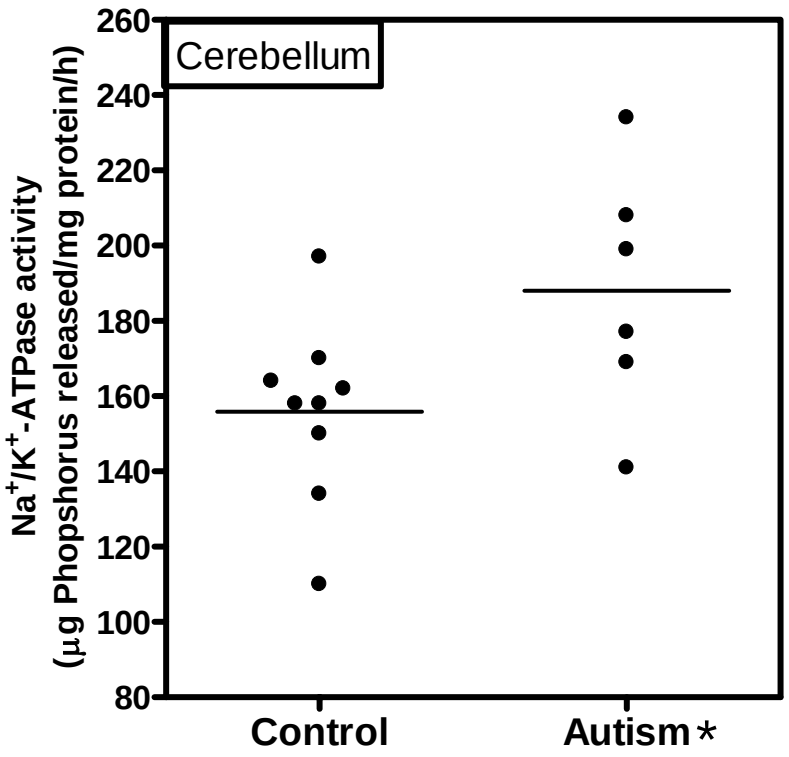

Fig. 1. Na^+/K^+ -ATPase activity in the cerebellum of autistic and control subjects.

The enzyme activity was measured in the cerebellum of autistic and control subjects as described in 'Materials and Methods'. The horizontal line represents average Na^+/K^+-ATPase activity in each group. * denotes $p < 0.05$, autism vs. control group.

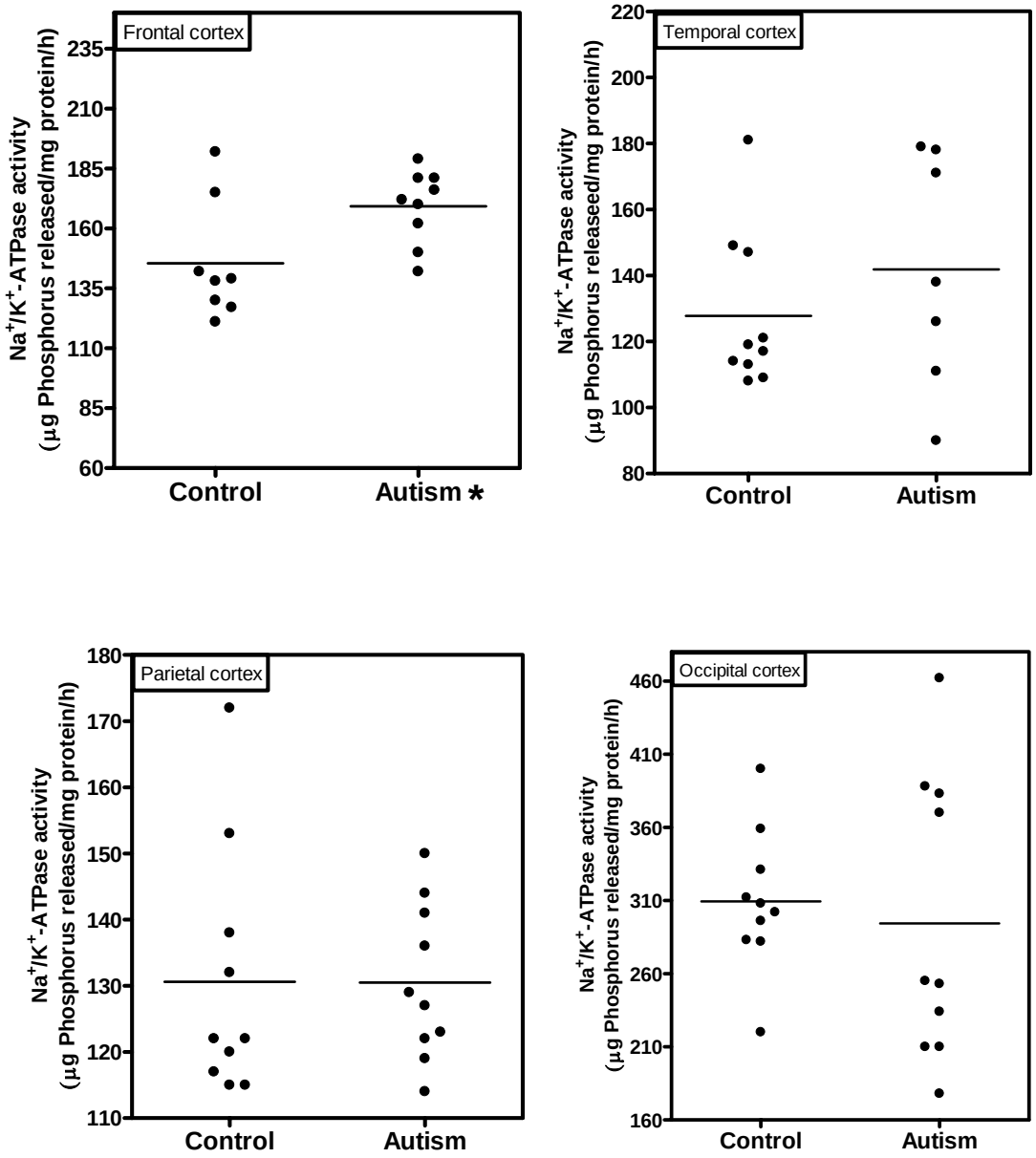

Fig. 2. Na^+/K^+ -ATPase activity in the frontal, occipital, parietal and temporal cortices from autistic and control subjects. The activity of Na^+/K^+-ATPase was measured in different brain regions from autistic and control subject as described in 'Materials and Methods'. * denotes $p < 0.03$, autism vs. control group.

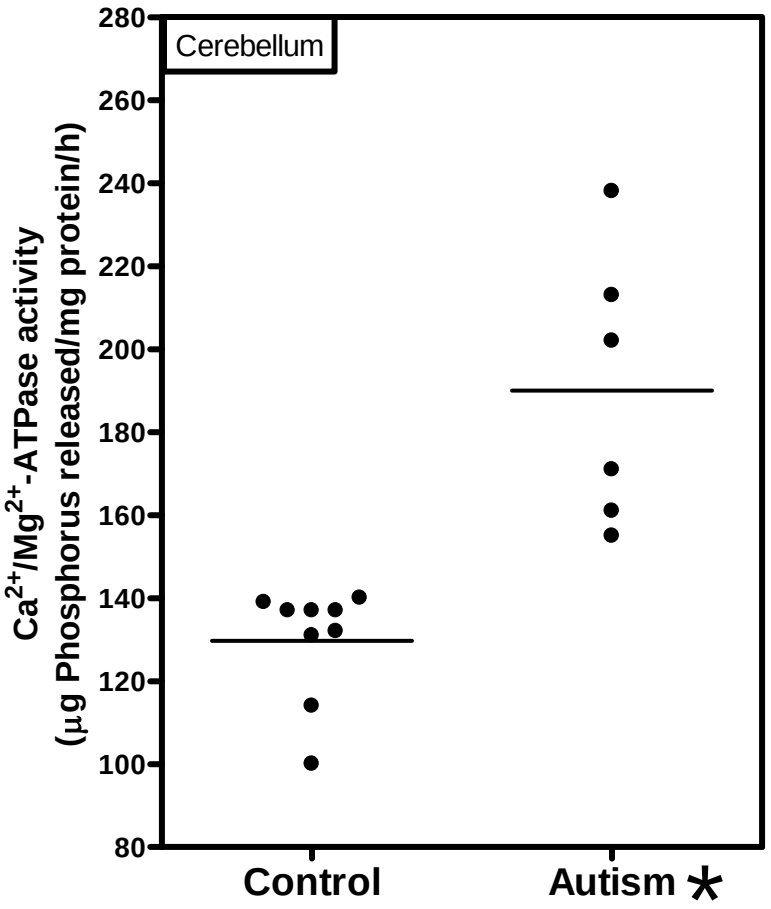

Fig. 3. Ca^{2+}/Mg^{2+}-ATPase activity in the cerebellum of autistic and control subjects. The enzyme activity was measured in the cerebellum of autistic and control subjects as described in 'Materials and Methods'. The horizontal line represents average Ca^{2+}/Mg^{2+}-ATPase activity in each group. * denotes $p < 0.0005$, autism vs. control group.

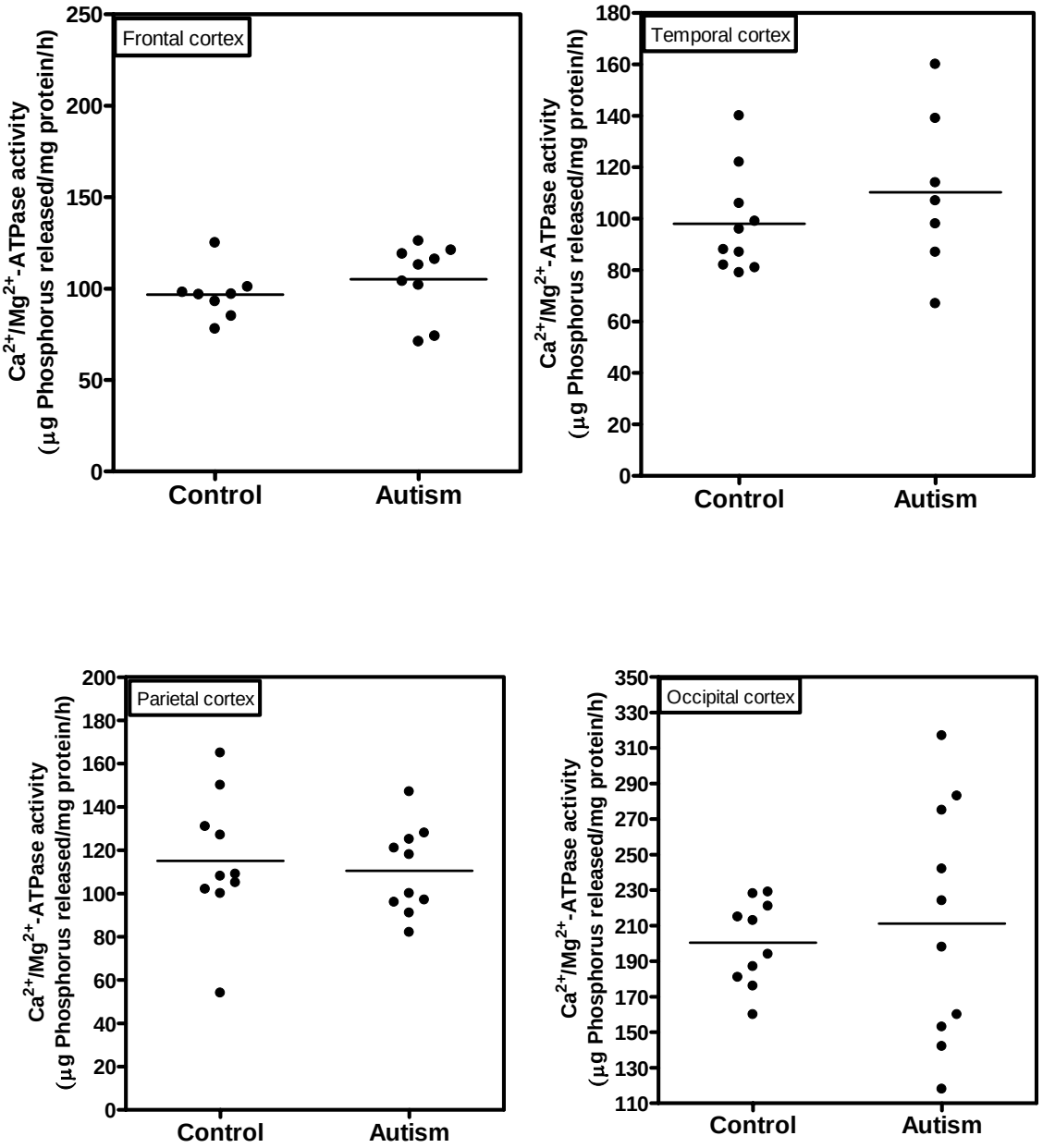

Fig. 4. Ca^{2+}/Mg^{2+}-ATPase activity in the frontal, occipital, parietal and temporal cortices of the brain regions from autistic and control subjects. The activity of Ca^{2+}/Mg^{2+}-ATPase was measured in different brain regions from autistic and control subjects as described in 'Materials and Methods'.